



Captopril modulates hypoxia-inducible factors and erythropoietin responses in a murine model of total body irradiation

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Objective. Our laboratory reported that the angiotensin converting enzyme inhibitor captopril improves erythroid recovery from total body irradiation (TBI) in mice when administered after irradiation. However, captopril administered before TBI attenuates erythroid recovery. Here we investigate captopril and radiation regulation of erythropoietin (EPO) and thrombopoietin (TPO), key effectors of erythroid progenitor proliferation and differentiation.

Materials and Methods. C57BL/6 mice, nonirradiated or exposed to 7.5 Gy TBI (⁶⁰Co, 0.6 Gy/min) were untreated or administered captopril. Plasma EPO and TPO levels were measured by enzyme-linked immunosorbent assay. Gene expression of EPO was determined by quantitative reverse transcription polymerase chain reaction. The hypoxia-inducible factors (HIF)-1 α and -2 α were measured by immunoblotting.

Results. In nonirradiated mice, continuous captopril administration in the water transiently reduced reticulocytes and red blood cells after 7 and 10 days, respectively. EPO plasma levels and gene expression were reduced below detectable limits after 2 days of captopril treatment, but recovered within 7 days. HIF-1 α and HIF-2 α were activated preceding reticulocyte and red blood cell recovery. TBI, which ablates early and late-stage erythroid progenitors, activated both HIFs and increased EPO and TPO. Captopril treatment postirradiation suppressed radiation-induced HIF activation and EPO expression. In contrast, captopril administration for 7 days before TBI resulted in earlier EPO induction and activation. Captopril treatment lowered TPO levels in nonirradiated mice, but had minimal effects on radiation-induced TPO.

Conclusions. In nonirradiated mice, captopril biphasically regulates EPO via HIF activation. TBI ablates erythroid progenitors, resulting in hypoxia, HIF activation, and increased EPO expression that are modulated by captopril treatment. These data suggest that short-term suppression of radiation-induced EPO immediately after TBI is favorable for erythroid recovery. © 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Angiotensin II (Ang II), a peptide hormone, is a key regulator of blood pressure and blood volume homeostasis [1–3]. More recently, it has been demonstrated that Ang II affects hematopoiesis [4–11]. Angiotensin converting enzyme (ACE) inhibitors, that prevent proteolytic processing of Ang II from Ang I and Ang II receptor antagonists reduced hematopoietic progenitor proliferation and maturation

in mice in vivo [12]. Ang II receptor antagonists reduced hemoglobin, hematocrit, and erythrocyte counts in rats [13]. In humans, administration of ACE inhibitors to patients or healthy volunteers transiently induced anemia [14–17]. In a case report, pancytopenia was attributed to ACE inhibition [18]. These findings indicate that Ang II modulators affect hematopoietic cell proliferation and development.

In vivo and in vitro studies have identified direct and indirect mechanisms for the effects of the Ang II peptide on hematopoiesis. Ang II directly induced proliferation of

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14. ABSTRACT

Objective. Our laboratory reported that the angiotensin converting enzyme inhibitor captopril improves erythroid recovery from total body irradiation (TBI) in mice when administered after irradiation. However, captopril administered before TBI attenuates erythroid recovery. Here we investigate captopril and radiation regulation of erythropoietin (EPO) and thrombopoietin (TPO), key effectors of erythroid progenitor proliferation and differentiation. **Materials and Methods.** C57BL/6 mice, nonirradiated or exposed to 7.5 Gy TBI (60Co, 0.6 Gy/min) were untreated or administered captopril. Plasma EPO and TPO levels were measured by enzyme-linked immunosorbent assay. Gene expression of EPO was determined by quantitative reverse transcription polymerase chain reaction. The hypoxia-inducible factors (HIF)-1a and -2a were measured by immunoblotting. **Results.** In nonirradiated mice, continuous captopril administration in the water transiently reduced reticulocytes and red blood cells after 7 and 10 days, respectively. EPO plasma levels and gene expression were reduced below detectable limits after 2 days of captopril treatment, but recovered within 7 days. HIF-1a and HIF-2a were activated preceding reticulocyte and red blood cell recovery. TBI, which ablates early and late-stage erythroid progenitors, activated both HIFs and increased EPO and TPO. Captopril treatment postirradiation suppressed radiation-induced HIF activation and EPO expression. In contrast, captopril administration for 7 days before TBI resulted in earlier EPO induction and activation. Captopril treatment lowered TPO levels in nonirradiated mice, but had minimal effects on radiation-induced TPO. **Conclusions.** In nonirradiated mice, captopril biphasically regulates EPO via HIF activation. TBI ablates erythroid progenitors, resulting in hypoxia, HIF activation, and increased EPO expression that are modulated by captopril treatment. These data suggest that short-term suppression of radiation-induced EPO immediately after TBI is favorable for erythroid recovery.

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specific hematopoietic lineages through angiotensin receptors on the surfaces of these cells [5–7]. Ang II induces proliferation of short term-hematopoietic stem cell (ST-HSC) and increases formation of myeloid progenitor colonies as measured by colony-forming units—granulocyte macrophage (CFU-GM) and colony-forming units—granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) colonies in culture [5]. Ang II also acts indirectly on hematopoiesis by modulating the production of erythropoietin (EPO), a potent kidney hormone that regulates the proliferation and differentiation of red blood cells (RBC) [4,19–22]. Other researchers demonstrated that ACE inhibitors reduced EPO plasma levels in healthy human subjects [23]. Ang II modulation of EPO production is hypothesized to occur via activation of angiotensin type I receptors and/or altered renal hemodynamics [24,25].

EPO and its receptor are potentially regulated under conditions of reduced oxygen saturation [26–28]. The hypoxia-inducible transcription factors (HIFs) provide a molecular link between EPO expression and low O₂ saturation, which can be triggered by a reduction of the hematocrit [29,30]. Active HIF is a heterodimer consisting of an oxygen-labile α -subunit and a constitutive β -subunit that binds to the hypoxia-response-element, an enhancer element in a responsive gene. Under normoxic conditions, HIF-1 α and HIF-2 α proteins are present in cells at low levels due to its degradation through the ubiquitin pathway. However, during hypoxia, HIF-1 α and HIF-2 α proteins are stabilized and form active complexes with the HIF-1 β isoform to enhance transcription of hypoxia-responsive genes, including EPO [31–33].

Ang II and ACE inhibitor administration modulates radiation-induced hematopoietic injury in vivo [34–37]. Our laboratory recently demonstrated in a murine model of total body irradiation (TBI) that the ACE inhibitor captopril can sensitize or mitigate hematopoietic radiation injuries. The effect depends on the time of captopril administration relative to radiation exposure [38]. Captopril given to mice before irradiation sensitized the hematopoietic system to injury but, treatment with captopril after radiation exposure resulted in increased survival and improved recovery of platelets, RBC, and reticulocytes [38]. We also determined that in nonirradiated mice captopril has a biphasic effect on the rate of proliferation of ST-HSCs [38]. Captopril administration for 2 consecutive days induced transient quiescence (increased G₀) of the ST-HSC population. However, after 7 consecutive days of administration, the ST-HSC population re-entered the cell cycle, indicating increased rates of proliferation. Therefore, captopril administration postirradiation induced ST-HSC quiescence, which correlated with improved hematopoietic recovery after radiation damage to the bone marrow. In the present study, we investigated the mechanism by which captopril effects erythroid responses in nonirradiated and irradiated mice.

Materials and methods

Mice

Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were 12 to 14 weeks of age (17.5–21.5 g) at the time of irradiation. Mice were housed four to five per cage in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal rooms were maintained at $21 \pm 2^\circ\text{C}$, $50\% \pm 10\%$ humidity, and 12-hour light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604) and acidified water (pH 2.5–3.0) to control opportunistic infections [39] were freely available. Animal handling procedures were performed in compliance with guidelines from the National Research Council and were approved by the Institutional Animal Care and Use Committee of the Armed Forces Radiobiology Research Institute (Bethesda, MD, USA).

Mice received 7.5-Gy TBI at a 0.6 Gy/minute dose rate in a bilateral gamma radiation field at Armed Forces Radiobiology Research Institute's ⁶⁰Co facility as described earlier [40]. We previously determined that 7.5 Gy TBI results in 50% lethality within 30 days (LD_{50/30}) for female C57BL/6J mice [41]. Captopril 0.55 g/L (USP grade; Sigma-Aldrich, St Louis, MO, USA) was dissolved in acidified water. An earlier study established captopril stability in acidified water [42]. Average captopril consumption was calculated based on volume of water consumed and body weight over the time course of the experiment. Nonirradiated mice had an effective dosage of 0.11 ± 0.01 mg/kg/day. In mice treated with captopril before irradiation, the average consumption was 0.10 ± 0.02 mg/kg/day. In mice treated with captopril after irradiation, the average consumption was 0.10 ± 0.05 mg/kg/day [38].

Experimental design

Experiment 1. The effects of captopril administration on blood cells, EPO, and HIFs were determined in nonirradiated mice untreated or administered captopril for 14 consecutive days. Levels of RBC and reticulocytes were determined on day 0 (baseline) and on days 2, 7, 10, and 14 after captopril treatment. Nucleated cell counts in the bone marrow were obtained on day 7. Plasma EPO and TPO, kidney EPO, and kidney HIFs were determined on days 0, 2, 7, and 10. **Experiment 2.** The effects of radiation and captopril administration on plasma EPO, kidney EPO, kidney HIFs, and liver angiotensinogen and EPO were examined on days 0, 2, 7, and 10 postirradiation. There were four treatment groups: untreated sham-irradiated mice (control); mice irradiated with the LD_{50/30} radiation dose (7.5 Gy); mice that received captopril for 7 consecutive days before 7.5-Gy irradiation; and mice that received 7.5 Gy and within 1 hour were placed on captopril treatment. For all experiments, separate groups of mice were used for all time points ($n = 4\text{--}6/\text{group}$).

Blood and bone marrow cell analysis

Mice were anesthetized using pentobarbital (10 mg/kg). Blood (0.5 mL) was collected by cardiac stick in EDTA-coated tubes to avoid clotting as described previously [41]. Complete blood counts with differentials were obtained within 30 minutes of blood collection using a Baker Advia 2120 Hematology Analyzer (Siemens, Tarrytown, NY, USA). Separate animals were used at each time point ($n = 5\text{--}6/\text{group}$). Femurs were surgically removed and cut at the ends. Bone marrow was flushed gently with cold

sterile phosphate-buffered saline and nucleated cells were counted [40].

EPO and angiotensinogen gene expression analysis

Mice were euthanized and kidneys and livers were placed in RNA-later (Qiagen, Valencia, CA, USA). On the same day, tissues were homogenized with an Ultra Thorax (Jahnke U. Kunkel, Staufen, Germany) in 0.5 mL RTL buffer (Qiagen). Samples were homogenized further by filtering through QIAshredder mini columns (Qiagen) for 3 minutes at 16,000g. RNA was isolated from 350 μ L filtrate using the RNeasy kit (Qiagen). Reverse transcription and quantitative real-time polymerase chain reactions were performed as described previously [43]. Primers were EPO forward 5'-TCT GCGACAGTCGAGTTCTG-3' and reverse 5'-CTTCTGCACAA CCC TCG T-3', giving a product size of 78 bp; angiotensinogen forward 5'-CGGAGGCAAATCTGAACAAC-3', reverse 5'-TCCT CCTCTCCTGCTTTGAG-3', giving a product of 94 bp. Data were collected from four to six replicates.

HIF-1 α and HIF-2 α protein levels

Kidneys were harvested and flash frozen on dry ice for storage at -80°C . For HIF-1 α , proteins were extracted in lysis buffer: 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 50 mM NaCl, 1m M EDTA, 50 mM HEPES, 1% Triton X-100 (adjusted to pH 7.5), with 2 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, and 1 tablet/10 mL buffer of complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). For HIF-2 α protein levels, nuclear extracts were prepared. Briefly, 140 mg tissue was homogenized on ice in 6 volumes of buffer (10 mM HEPES, 7.8; 10 mM KCl; 0.1 mM EDTA; 1.5 mM MgCl_2 ; 0.2% NP-40; 0.5 mM dithiothreitol; 1 mM Na_3VO_4 ; protease inhibitor [Roche]). Nuclei were pelleted at 3000 rpm in a microcentrifuge for 10 minutes at 4°C . The pellet containing nuclei were resuspended in 0.1 mL nuclear extraction buffer (20 mM HEPES, 7.8; 420 mM NaCl; 0.1 mM EDTA; 1.5 mM MgCl_2 ; 20% glycerol; 0.5 mM dithiothreitol; 1 mM Na_3VO_4 ; protease inhibitor), vortexed for 10 minutes, and centrifuged for 10 minutes at 12,000 rpm. To the supernatant, 2.5 U Benzonase (Novagen, Madison, WI, USA) was added and incubated for 20 minutes on ice. Proteins (70 μ g) were resolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to Western blot using anti-HIF-1 α antibody (1:1000; Novus Biological, Littleton, CO, USA) or with anti-HIF-2 α antibodies (1:200 dilution of a 1:1 mixture of #NB100-122; Novus Biological and #199; Abcam, Cambridge, MA, USA) as described previously [43].

EPO and TPO plasma levels

Plasma EPO and TPO were determined using the Quantikine mouse/rat immunoassays (R&D Systems, Minneapolis, MN, USA). Plasma was diluted 1:2 for EPO determination or 1:5 for TPO determination using diluent provided by the manufacturer. Duplicates of plasma samples, standards (EPO standards, 47–3000 pg/mL; TPO standards, 62.5–4000 pg/mL), and controls were with monoclonal antibodies for 2 hours. Plates were read using a MTX TC Microplate Reader (Dynex, Chantilly, VA, USA).

Statistical analysis

Plasma EPO and TPO, EPO messenger RNA (mRNA) levels, and hematology results were analyzed using one-way analysis of variance followed by Holm-Sidak's or Tukey's using SigmaStat 3.1 (Point Richmond, CA, USA). A value of $p \leq 0.05$ (two-tailed) was considered statistically significant.

Results

Captopril administration biphasically modulates levels of circulating RBC, reticulocytes, serum EPO, and kidney EPO, HIF-1 α , and HIF-2 α expression in nonirradiated mice

Our laboratory recently reported that captopril administration modulates reticulocyte and RBC recovery after TBI in mice [38]. We investigated captopril effects on blood cells, EPO, and HIF activation in nonirradiated mice that received captopril for 2 to 14 consecutive days. Blood cell analysis showed that RBC were reduced after 10 days of captopril administration (control, $8.7 \pm 0.3 \times 10^6$ cells/ μ L; captopril, $7.8 \pm 0.2 \times 10^6$ cells/ μ L; $p < 0.05$). RBC and reticulocyte levels returned to basal levels by day 14 (Fig. 1A). Reticulocytes were reduced after 7 and

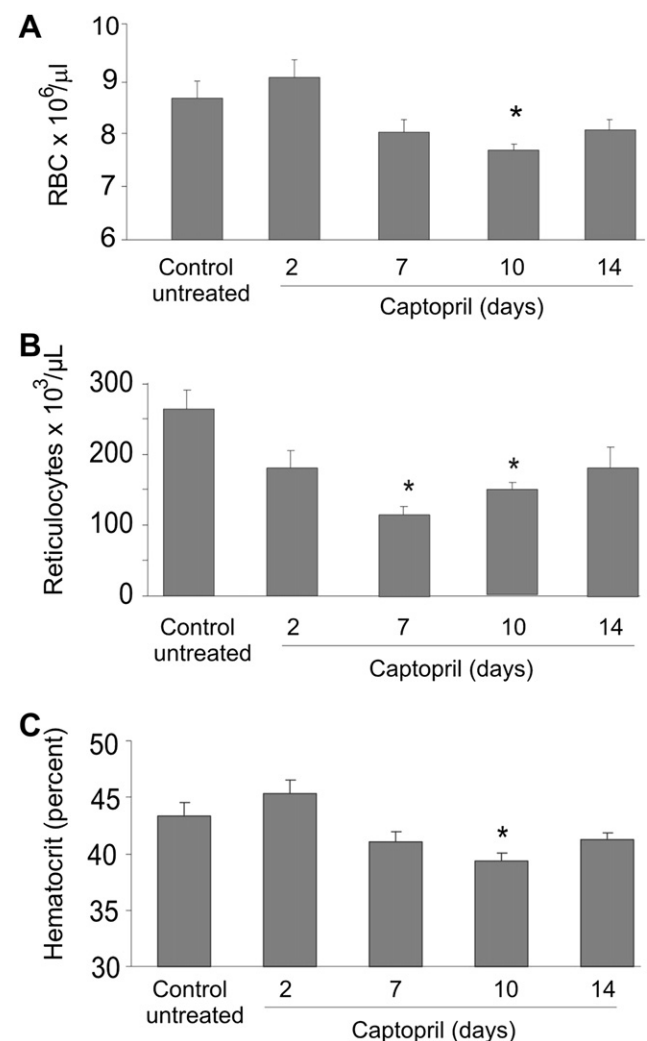


Figure 1. Effect of captopril administration on peripheral blood erythroid cells. Nonirradiated mice were continuously administered captopril in their drinking water for the indicated times. (A) Peripheral RBCs, (B) reticulocytes, and (C) hematocrit were determined. Data show mean values \pm standard error of mean ($n = 5$ –6 mice/group). * $p < 0.05$ compared to basal blood cell levels.

10 days of captopril treatment (control, $257 \pm 22 \times 10^3$ cells/ μ L; captopril, 7 days $112 \pm 12 \times 10^3$ cells/ μ L; $p < 0.05$; captopril, 10 days $148 \pm 9 \times 10^3$ cells/ μ L; $p < 0.05$) (Fig. 1B). Hematocrit was reduced after 10 days of captopril treatment (control, $43\% \pm 1.2\%$; captopril 7 days, $39\% \pm 1.6\%$; $p < 0.05$) (Fig. 1C).

After 2 days of captopril administration, plasma EPO levels fell from 82 ± 12 pg/mL (control) to below the level of detection (<25 pg/mL; $p < 0.05$ from control) (Fig. 2A). Within 7 days of captopril administration, the level of EPO recovered to 71 ± 6 pg/mL, and by day 10 the average EPO was significantly above basal levels (200 ± 110 pg/mL; $p < 0.05$ from control). The observed alterations in EPO protein levels in the plasma were also reflected in the biphasic regulation of EPO mRNA levels in the kidney (Fig. 2B). We did not find any EPO expression in the liver over the entire time course of the experiment (data not shown).

HIF-1 α and HIF2- α protein levels are increased in conditions of reduced oxygen saturation and upregulate a variety of hypoxia response genes, including EPO [44-46]. We investigated the levels of HIF-1 α and HIF-2 α in the kidneys (Fig. 3). HIF-1 α protein increased more than twofold above basal levels after 7 days of captopril treatment (Fig. 3). HIF-2 α protein increased twofold after 2 and 7 days of captopril treatment, returning to baseline after 10 days of captopril administration.

Because EPO and Ang II stimulate development and proliferation of hematopoietic progenitors, we evaluated the effect of captopril administration on constitutive hematopoiesis in the marrow compartment. Seven consecutive days of captopril treatment did not significantly alter the nucleated cellularity of the bone marrow compared with control mice. This indicated that there was no gross defect in total hematopoietic cell production in the marrow (Fig. 4A), although significant increases in myeloid progenitors (CFU-GM, CFU-GEMM, and colony-forming units–myeloid [CFU-M]) were detected, with no significant change in erythropoietic burst formation activity (Fig. 4B).

Radiation-induced plasma EPO expression and HIF-1 α and HIF-2 α activation are modulated by captopril

We demonstrated that captopril administration for 7 consecutive days before irradiation sensitized animals to radiation-induced hematopoietic injury. In contrast, captopril treatment starting the day of irradiation and continuing for 30 consecutive days postirradiation was protective against hematopoietic damage [38]. Hematocrit levels were severely reduced after radiation exposure from basal levels ($43.3\% \pm 0.6\%$) by radiation at days 7, 10, and 14 postirradiation ($31.7\% \pm 0.6\%$, $26.1\% \pm 1.4\%$, and $14.7\% \pm 0.6\%$, respectively; $p < 0.05$ from control) (Fig. 5A). Reductions in hematocrit levels by radiation were modulated by both captopril regimens. Pretreatment with captopril exacerbated the reduction in hematocrit ($38\% \pm 0.8\%$, $27.2\% \pm$

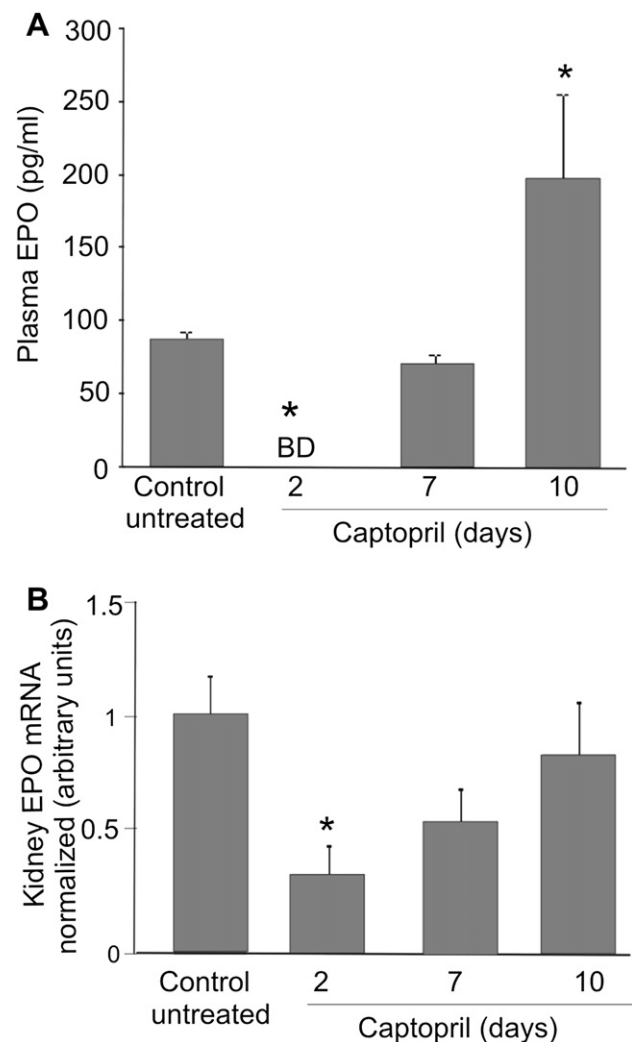


Figure 2. Captopril administration biphasically modulates erythropoietin in nonirradiated mice. Nonirradiated mice were continuously administered captopril in their drinking water for the indicated times. (A) Plasma EPO levels were determined by enzyme-linked immunosorbent assay. Measurements were performed in duplicate for each sample. Graph shows the means \pm standard error of mean of four mice per group at each time point. BD = below detection (<25 pg/mL). * $p < 0.05$ compared with basal levels of EPO. (B) EPO expression levels in the kidney were determined by quantitative reverse transcription polymerase chain reaction. Expression was determined in triplicate for each sample and normalized as described in the Materials and Methods. Graph shows the mean \pm standard error of mean of four mice per group at each time point. * $p < 0.05$ compared with basal expression of EPO.

0.6% , $17.9\% \pm 0.7\%$, and $9.2\% \pm 0.4\%$ on days 2, 7, 10, and 14 postirradiation, respectively; $p < 0.05$ from radiation alone). In contrast, treatment with captopril postirradiation lessened the reduction in hematocrit, compared with mice that received radiation alone ($30.6\% \pm 0.7\%$, and $24.7\% \pm 1.0\%$ on days 10 and 14 postirradiation, respectively; $p < 0.05$ from radiation alone).

We examined the effects of radiation alone and in combination with both regimens of captopril on EPO

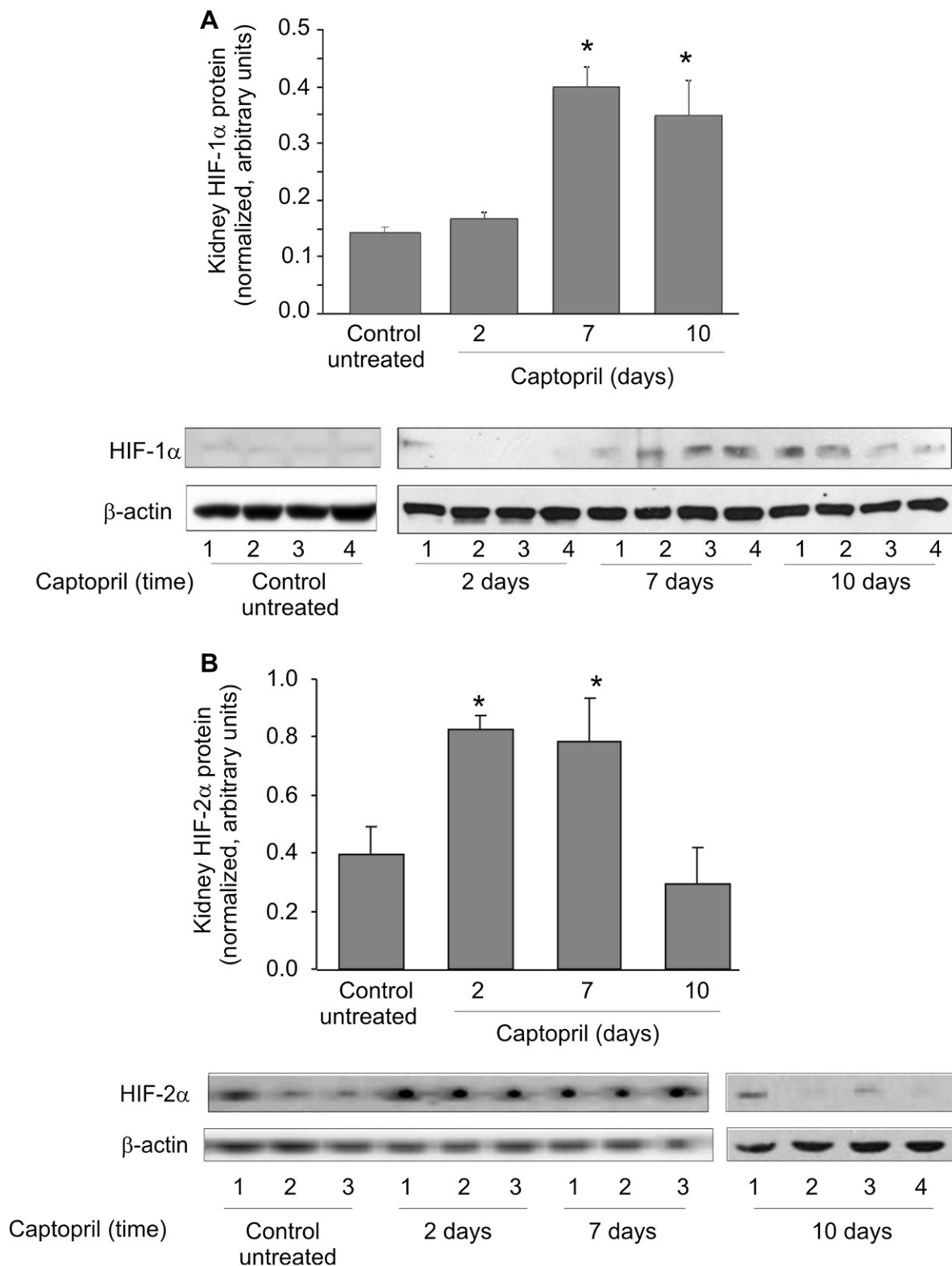


Figure 3. Captopril administration modulates HIF-1 α and HIF-2 α protein levels in nonirradiated mice. Nonirradiated mice were either untreated or continuously administered captopril in their drinking water. **(A)** HIF-1 α protein levels were determined by Western blotting whole-cell lysates from kidneys at the indicated time points. **(B)** HIF-2 α protein levels were determined by Western blotting nuclear extracts from kidneys at the indicated time points. HIF protein levels were normalized to β -actin, and standards were used to normalize results between gels. Each value represents the mean \pm standard error of mean of three to four mice per group at each time point. * p < 0.05 compared with basal levels of HIF-1 α and HIF-2 α .

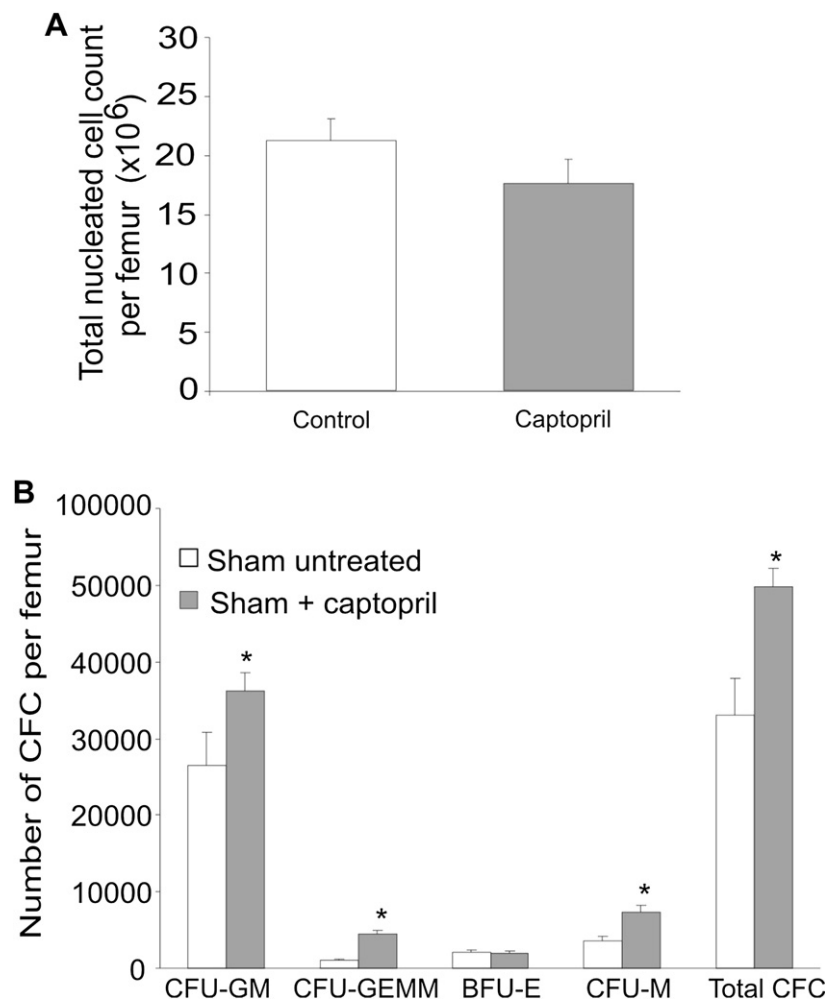


Figure 4. Effect of captopril on nucleated bone marrow cell counts and specific lineage progenitors in nonirradiated mice. Mice received either no captopril (control) or were treated with captopril (0.1 mg/kg/day) for 7 consecutive days. At day 7, femoral bone marrow cells were collected. (A) Total numbers of nucleated cells from the bone marrow. (B) Specific lineage colony-forming cells were determined for multipotential (CFU-GEMM), myeloid (CFU-GM, CFU-M), and erythroid (BFU-E). For both panels, results represent mean \pm standard error of mean of eight mice. * $p < 0.05$, significant difference compared with control group.

plasma levels and EPO mRNA expression. Radiation alone increased plasma EPO levels compared with controls at days 2 to 10 postirradiation (control, 87 ± 12 pg/mL; day 2 postirradiation, 326 ± 90 pg/mL; day 7 postirradiation $6,620 \pm 1830$ pg/mL; and day 10 postirradiation $15,460 \pm 6,560$ pg/mL ($p < 0.05$ at all time points) (Fig. 5B). Changes in EPO plasma levels were reflected in increased levels of EPO mRNA in the kidney (Fig. 5C). No EPO mRNA expression was detected in the liver under any of the treatment conditions at any time point (data not shown).

Administration of captopril to mice for 7 days before irradiation resulted in a greater increase in EPO levels compared with animals that received radiation alone. EPO levels at 2 and 7 days postirradiation were $3,020 \pm 340$ pg/mL and $13,280 \pm 1,220$ pg/mL, respectively (Fig. 5B, C). At day 10, plasma EPO levels in the captopril pretreatment group were similar to those in mice treated with radiation alone, although EPO mRNA in the kidney

increased at this time point (Fig. 5B, C). In contrast, captopril treatment postirradiation suppressed radiation-induced plasma EPO compared with radiation alone (Fig. 5B). Suppression was significant at all days: day 2: 20 ± 4 pg/mL; day 7: $1,630 \pm 60$ pg/mL; and day 10: $4,590 \pm 1520$ pg/mL ($p < 0.05$ for all times compared with radiation alone).

We next examined HIF protein activation. Radiation alone significantly activated kidney HIF-1 α on days 2, 7, and 10 postirradiation (2.5-fold, 6-fold, and 7-fold, respectively; Fig. 5D). Radiation also activated HIF-2 α , significant ($p < 0.05$) on days 7 and 10 postirradiation (2-fold and 4.5-fold, respectively; Fig. 5E). In mice administered captopril before irradiation, HIF-1 α was induced to a greater extent than by radiation alone at all time points (2.2-fold, 2.2-fold, and 2.6-fold, respectively) (Fig. 5D). In mice pretreated with captopril, HIF-2 α was increased sixfold compared to radiation alone on day 2 postirradiation, but

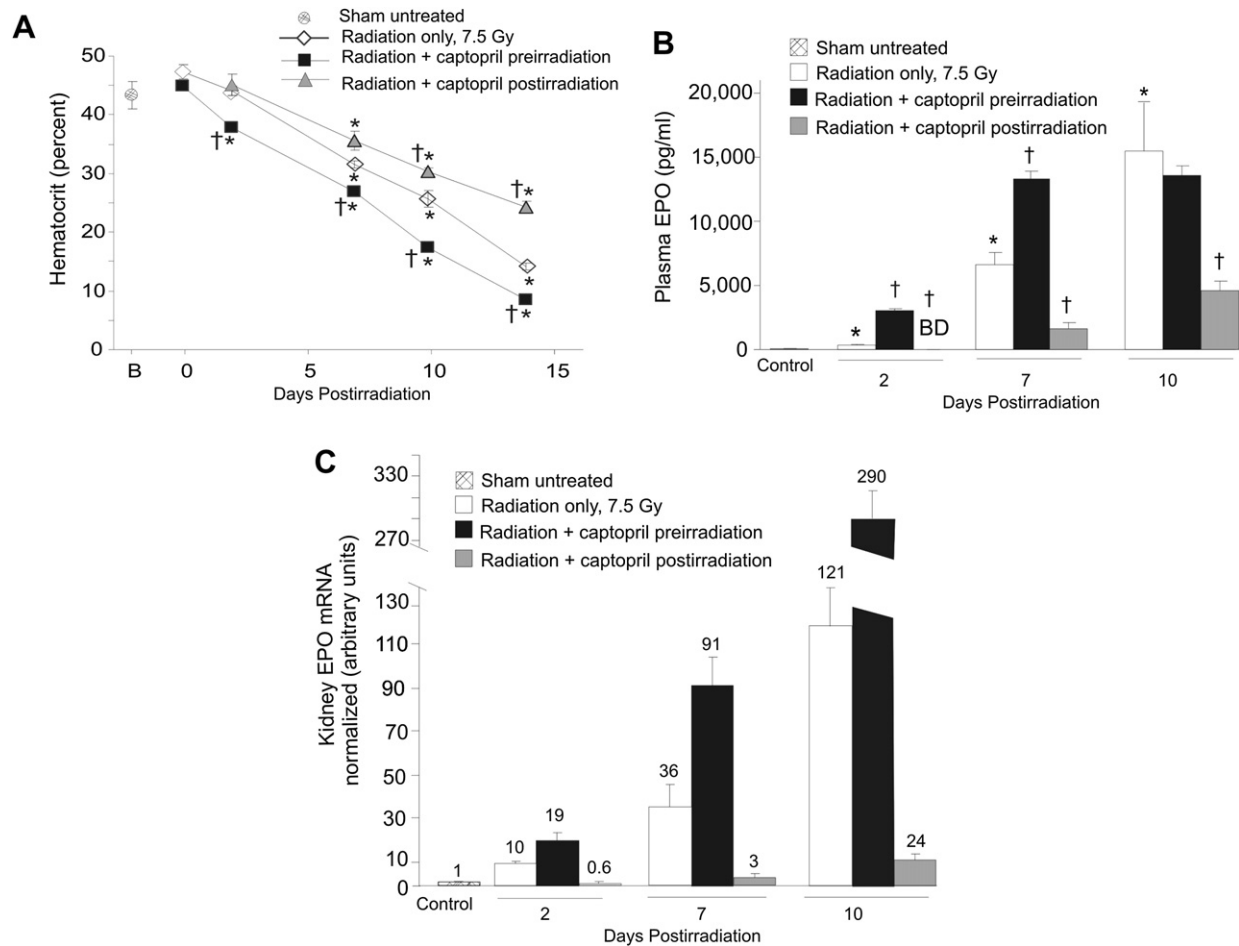


Figure 5. Captopril administration affects radiation-induced reduction in hematocrit, and production of EPO HIF-1 α , and HIF-2 α . (A) Peripheral blood hematocrit (HCT); (B) plasma EPO from peripheral blood; (C) kidney EPO mRNA;

by day 7 postirradiation HIF-2 α was equal to levels induced by radiation alone, and by 10 days, HIF-2 α was approximately twofold lower. The treatment of mice with captopril postirradiation substantially suppressed radiation-induced HIF-1 α and HIF-2 α at all time points (Fig. 5D, E).

Because Ang II also modulates EPO levels [19,23,25], we investigated the regulation of angiotensinogen expression by radiation as an alternative mechanism of radiation-induced increase of EPO levels. The liver is a primary source of plasma angiotensinogen that is regulated primarily via gene expression [47]. Quantitative polymerase chain reaction analysis showed that radiation did not significantly alter the levels of angiotensinogen expression in the liver at 2 or 7 days postirradiation (Fig. 6), suggesting that regulation of angiotensinogen is not the mechanism for radiation elevation of EPO levels at early time points. However, after 10 days, angiotensinogen levels were ~1.8-fold above baseline levels in response to radiation, suggesting that Ang II may contribute to radiation-induced EPO levels at later time points. Angiotensinogen levels were not significantly changed by captopril administration, except at day 10 in mice treated with captopril before irradiation.

Captopril does not modulate radiation effects on plasma TPO levels

TPO, a cytokine that potently regulates platelet production, also stimulates survival and proliferation of late erythroid progenitors [48,49]. We examined whether captopril and/or radiation could regulate this cytokine in addition to the regulation of EPO. Continuous administration of captopril to nonirradiated mice resulted in ~40% reduction of plasma TPO levels by day 10 (Fig. 7). In contrast, TPO was induced 150% by radiation at days 7 and 10 postirradiation. Administration of captopril before irradiation resulted in a reduction in radiation-induced TPO production only on day 7 postirradiation. Radiation-induced TPO production was not affected by captopril treatment postirradiation.

Discussion

The primary finding of this study is that captopril treatment modulates radiation-induced EPO production and HIF hypoxia responses. Captopril administration before radiation exposure exacerbated radiation-induced HIF activation and EPO expression, whereas captopril treatment after radiation

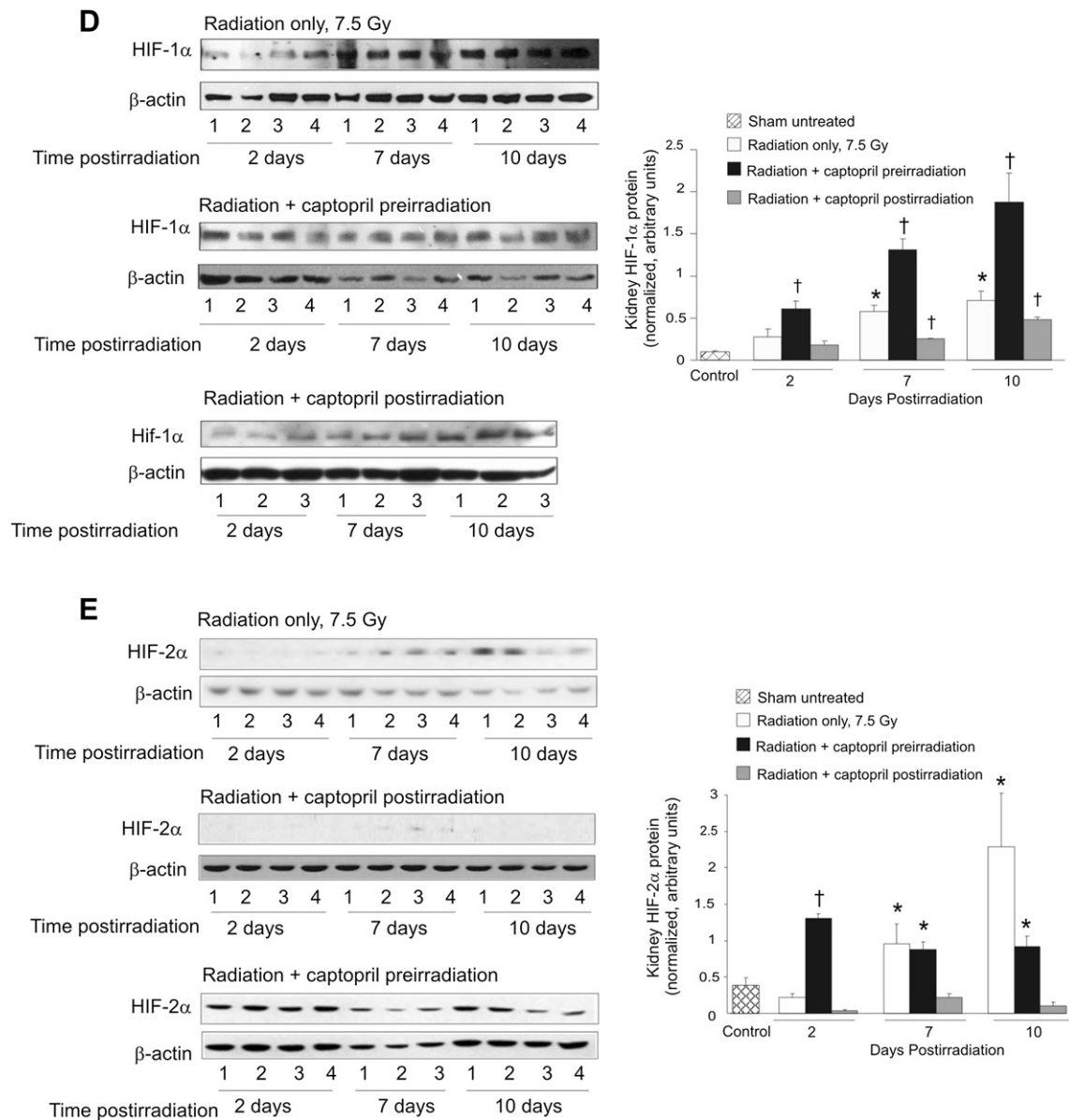


Figure 5. (continued) and (D, E) HIF-1 α protein (D) and HIF-2 α protein (E) levels were determined in the following groups: 1) nonirradiated controls; 2) 7.5-Gy irradiated mice with no treatment; 3) 7.5-Gy irradiated mice pretreated with captopril for 7 days before irradiation (days -7 to 0); and 4) 7.5-Gy irradiated mice treated with captopril postirradiation (days 0 to +10). Samples were obtained for the indicated times postirradiation. Each value represents the mean \pm standard error of mean of three to four mice per group at each time point. BD = below detection (<25 pg/mL). * $p < 0.05$ compared with basal levels of EPO, HIF-1 α , or HIF-2 α ; † $p < 0.05$ compared with radiation alone at the same time point.

exposure suppressed these responses. These findings provide a potential mechanism for previous studies from our laboratory demonstrating that captopril administration modulates the recovery RBC and reticulocytes after ^{60}Co TBI in mice. Captopril given before irradiation resulted in accelerated loss of RBC and reticulocytes, whereas captopril administration after irradiation spared RBC and reticulocytes [38].

In nonirradiated mice, captopril administration biphasically modulated RBC and reticulocyte levels, with nadirs after 7 to 10 days of captopril treatment and recovery between 10 and 14 days of captopril treatment. Others

have demonstrated Ang II modulates EPO production under homeostatic conditions [25,50]. We found that the time course of the biphasic effect on RBC and reticulocytes correlated with biphasic effects on EPO plasma levels and EPO mRNA levels in the kidney. Captopril resulted in an initial reduction of EPO plasma levels to below detection limits within 2 days followed by an increase in EPO plasma at 7 and 10 days of captopril administration. Our data suggest that the delayed recovery of EPO gene expression and plasma EPO may be driven at least in part by HIF activation in nonirradiated mice.

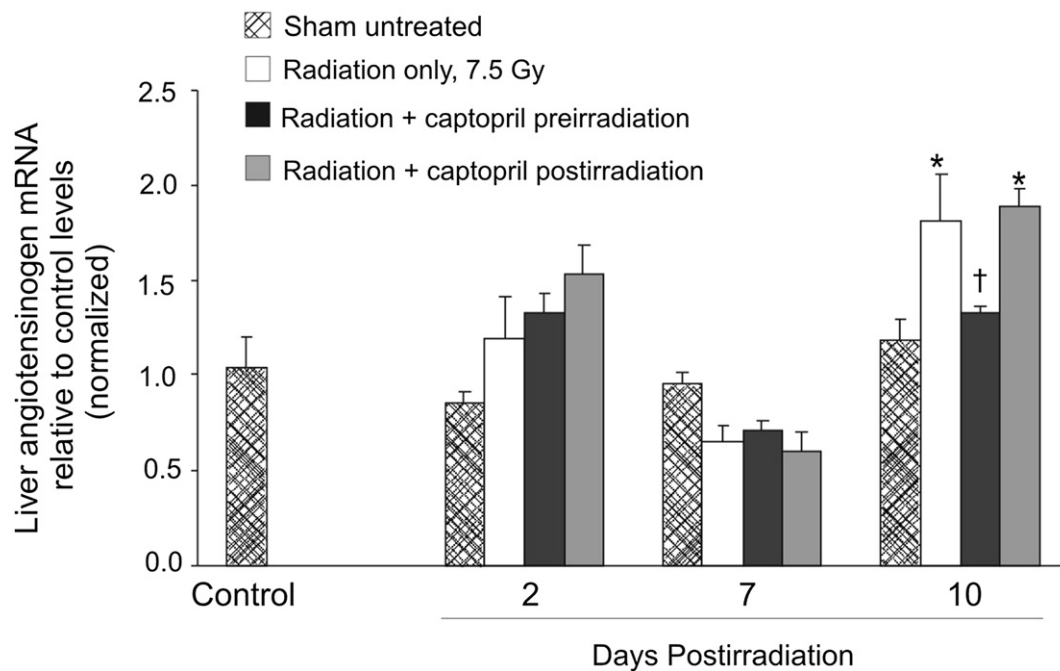


Figure 6. Radiation effects on angiotensinogen expression in mice liver. Quantitative polymerase chain reaction analysis of angiotensinogen mRNA in the liver of nonirradiated controls and 7.5-Gy irradiated mice with no treatment. Each value represents the mean \pm standard error of mean of four mice per group at each time point. * $p < 0.05$ compared with basal levels of nonirradiated mice; † $p < 0.05$ compared with radiation alone at the same time point.

Radiation exposure resulted in the activation of HIFs and increased EPO expression within 2 days postirradiation. As stated here, captopril administration for 7 days in nonirradiated mice induced HIF activation and EPO expression. Previous studies have shown that ionizing radiation activates HIF-1 α , perhaps through generation of reactive oxygen species [51]. When radiation exposure occurred after 7 days of captopril administration, there was an augmentation of radiation-induced HIF activation and EPO production. We hypothesize that captopril administration for 7 days before irradiation predisposed the mice to exaggerated hypoxia and EPO responses after radiation exposure. One exception to this enhancement was observed on day 10 postirradiation, when plasma EPO levels in the captopril pretreatment group were similar to those in mice treated with radiation alone. The absence of an increase in plasma EPO may be due to a variety of pathological conditions, such as multiorgan failure. However, when captopril was administered after irradiation, we observed a suppression of radiation-induced hypoxia, including a decrease in radiation-induced HIF activation and EPO expression. The mechanism for this suppression remains unclear. It is possible that at early time points after captopril administration, reduction of systemic blood pressure might increase kidney perfusion, thus delaying activation of HIFs by radiation.

As stated, we previously showed that administration of captopril for 7 days before TBI exacerbated the ablation of RBC and reticulocytes [38]. It is seemingly paradoxical that increased EPO production with captopril administra-

tion before irradiation results in accelerated loss of RBC and reticulocytes. We hypothesize that the differential effects of captopril on radiation-induced RBC and reticulocyte ablation are related to the time course of effects of captopril on EPO expression. EPO promotes survival, proliferation, and differentiation of erythroid progenitor/precursor cells [22,52]. Erythropoietic burst-forming units express the EPO receptor but do not require EPO for survival/proliferation. EPO has not been found to augment the mobilization of hematopoietic stem cells, but instead is considered a late-acting hematopoietic growth factor [53]. In nonirradiated mice, administration of captopril for 7 days induced expression of EPO, likely resulting in increased proliferation in EPO-responsive hematopoietic progenitors. Radiation studies indicate that rapidly proliferating cells are more susceptible to radiation-induced damage [54]. Therefore, induction of the growth of specific hematopoietic lineages by EPO before radiation exposure would likely predispose those specific cells to increased damage by radiation. In contrast, captopril treatment postirradiation dampens radiation-induced EPO expression. Recently, we reported that a 2-day captopril treatment in nonirradiated mice caused a transient pause in the *in vivo* cell cycling of marrow Lin⁻ and Lin⁻ Sca-1⁺ ckit⁺ hematopoietic progenitor cells [38]. A pause in cell cycling after high-dose irradiation may aid in DNA repair, cell survival, and hematopoietic cell recovery [54].

We previously observed that captopril treatment postirradiation enhanced recovery of platelets [38]. Our current findings indicate that in nonirradiated animals, captopril

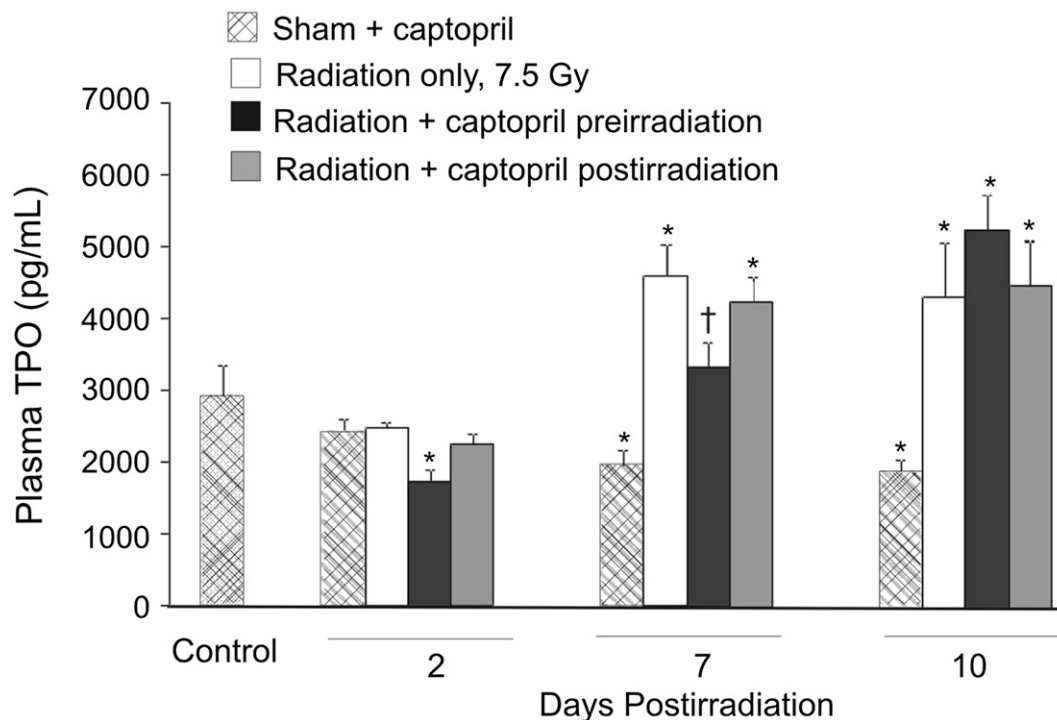


Figure 7. Comparison of plasma TPO levels in mice treated with different regimen of captopril. TPO levels was measured in the plasma of nonirradiated controls, 7.5-Gy irradiated mice with no treatment, 7.5 Gy-irradiated mice pretreated with captopril for 7 days before irradiation (days -7 to 0) and 7.5 Gy irradiated mice treated with captopril post irradiation (days 0 to +10). Samples were obtained at the indicated times postirradiation, and measurements were performed in triplicate for each sample. Each value represents the mean \pm standard error of mean of four mice per group at each time point. * $p < 0.05$ compared with basal levels of TPO (sham); † $p < 0.05$ compared with the radiation-induced TPO level at the same time point.

treatment increased marrow cellularity and CFU-GEMM, CFU-GM, and CFU-M progenitor cell content. Based on these findings, we hypothesize that captopril treatment may affect TPO regulation, the primary cytokine controlling megakaryocyte proliferation. TPO influences the growth of stem/progenitor cells capable of long-term multilineage hematopoietic repopulation [55]. In nonirradiated mice, captopril significantly suppressed TPO levels in the plasma. However, captopril did not significantly alter radiation-induced TPO, suggesting that TPO regulation is not the mechanism by which captopril modulates radiation effects on hematopoiesis. The increase of bone marrow multipotent and myeloid progenitor cells suggests active hematopoiesis may be a compensatory hematopoietic mechanism that correlates with the increased erythroid cell loss/imbalance and erythroid demand with captopril-treatment.

ACE inhibitors are being used extensively for treatment of breast cancer, leukemia, and lymphoma to prevent cardiac complications associated with chemotherapy regimens [56]. Because these patient populations may also undergo radiation treatment, there is a requirement for improved understanding of the potential effects of ACE inhibitors on radiation-induced injuries. Captopril and other ACE inhibitors have been demonstrated to modify a variety of late radiation tissue injuries, including kidney, lung, skin,

and heart [57]. More recently, EPO receptors have been identified on a variety of cells, including endothelial cells, neuronal cells, cardiac myocytes, and vascular smooth muscle cells [52,58], although the functions of these receptors are still under investigation. It remains to be determined whether mitigation of radiation injury by ACE inhibitors in other organs is the result of inhibition of Ang II or whether indirect regulation of other cytokines or factors, such as EPO, is also involved.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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